should be noted that the 55-year-old patient has electoretinographic responses of at least 1 μV, has visual fields with remaining peripheral crest, has marked sparing of the macular region, and still retains useful vision. This is in contrast to a family we recently described with a deletion of exons 8 to 10. In that family no electoretinographic responses were detected in an individual 40 years of age, and even the carriers were visually handicapped. XLRP phenotypes caused by a few other mutations in the RPGR gene have been described recently. With these and other similar descriptions, it is expected that a clear genotype-phenotype relationship pattern will begin to emerge in the near future.

Most RPGR mutations seem to have originated independently, as suggested by four different mutations found in Swedish XLRP families (and in this report). Along these lines, we should note that a total of 15 different RPGR mutations were identified in 17 families during the analysis of 80 independent XLRP families. These results suggest that most XLRP families have different RPGR mutations and are usually not a result of a predominant mutation or mutations or a founder effect in our XLRP population.

Acknowledgment
The authors thank Ing-Marie Holst for skillful technical assistance.

References

Retrovirus-Mediated Gene Transfer to Photocoagulation-Induced Choroidal Neovascular Membranes
Toshinori Murata,1 Masanori Hangai,1 Tatsuro Ishibashi,2 Christine Spee,3 Erlinda M. Gordon,3 W. French Anderson,3 David R. Hinton,4 and Stephen J. Ryan1

From the 1Department of Ophthalmology, Doheny Eye Institute, the 2Department of Pediatrics and Biochemistry, and the 4Department of Pathology, University of Southern California School of Medicine, Los Angeles; and the 3Department of Ophthalmology, Faculty of Medicine, Kyushu University, Fukuoka, Japan.

Supported in part by Grants EYO1545 and EYO0400 from the National Institutes of Health (Bethesda, Maryland) and by The Hoover Foundation (Pasadena, California). The Department of Ophthalmology (University of Southern California School of Medicine, Los Angeles) is the recipient of an unrestricted award from Research to Prevent Blindness, New York, New York.

Submitted for publication February 18, 1998; revised June 5, 1998; accepted July 9, 1998.

Proprietary interest category: C5.

Reprint requests: Stephen J. Ryan, Doheny Eye Institute, 1450 San Pablo Street, Los Angeles, CA 90033.

PURPOSE. To determine the feasibility of experimental gene transfer to laser-induced choroidal neovascular membrane (CNVM) in rats, with a retroviral vector containing the reporter construct β-galactosidase (β-gal).

METHODS. Laser photocoagulation was used to induce CNVM in rats. To ascertain the duration of β-gal expression in the CNVM, 23 rats received 10 burns (75 mJ, 100 mW, 0.1 seconds) in their right eyes, and β-gal expression was examined from day 3 to 4 months. In addition, 14 pigmented rats were treated with 3 photocoagulation burns in their right eyes. β-gal vector was injected into the vitreous or subretinal space 2 days later. On day 14, fluorescein angiography was performed to detect choroidal neovascularization. Then, β-gal expression in each photocoagulation-induced CNVM was examined by observing the exposed fundus of the eyes stained with the β-gal substrate X-Gal.

RESULTS. β-gal expression was identified in the CNVM induced by photocoagulation from day 5 (16.2% ± 6.8% of the lesions) to 4 months (3.7% ± 2.4%). Histopathologic examination revealed β-gal-transduced macrophages and spindle-shaped cells, which amounted to 1.12% ± 0.58% (at 2 weeks) of the total cells in the CNVM. β-gal expression was restricted to the CNVM, and there was no β-gal transduction in surrounding normal retinochoroidal tissue. There was no correlation between choroidal neovascularization formation and β-gal expression.

Age-related macular degeneration (AMD) is a major cause of acquired blindness as a result of the development of choroidal neovascularization (CNV) associated with overlying retinal damage. New therapies, including thalidomide, radiation, interferon α-2a, and surgical removal of the CNVM, have been tested, but as of this time they have been relatively unsuccessful. A novel approach to this problem would be to use gene therapy to produce local long-term expression of anti-angiogenic factors in the subretinal space. For this purpose, the demonstration of the feasibility of this approach in a reproducible animal model is necessary. Laser-induced CNV models have been widely used to investigate the natural course of CNV, to compare fluorescein angiograms and histologic findings of the same lesions, and to determine the expression of angiogenic factors in the process of CNV evolution. Vascular endothelial growth factor and basic fibroblast growth factor, identified in surgically excised CNVM from eyes with AMD, also have been localized in photocoagulation-induced CNVM. This model also has been used to determine the possibility of pharmacologic treatment and photodynamic therapy for CNV.

The vector of choice for gene delivery to ocular tissues depends on the cells to be targeted and the length of time that expression is required. To treat CNV, long-term expression of transferred genes must be achieved to obtain therapeutic benefits because AMD is chronic in nature. To transfer exogenous genes into retinohemoidal tissue, various vectors have been used including adenovirus vectors, adeno-associated virus vectors, herpes virus vectors, human immunodeficiency virus-based lentiviral vectors, and retroviral vectors. Among these vectors, only retroviral vectors and human immunodeficiency virus-based lentiviral vectors can provide long-term expression of transferred genes, because they insert exogenous genes into the host chromosomal DNA. Adenoviral vectors have been most commonly used in experimental gene transfer to ocular tissue. However, they may not be suitable for gene therapy to treat AMD, because the expression of the transferred genes is transient. This is probably due to an immune response to the adenovirus. Herpes viral vectors may not be suitable because they show evidence of cytoxicity. Retroviral vectors have been the carrier of choice in the majority of clinical human gene therapy trials, and they have been found to be generally safe with no unexpected toxicities described. Because of these features, we believe, at this time, that retroviral vectors are the vector of choice for delivering anti-angiogenic genes to the CNVM.

The aim of the present study is to determine the feasibility of using retroviral vectors to target the gene transfer to the CNVM induced by photocoagulation. For this purpose, we evaluated the capacity of a retroviral vector to transduce the β-gal gene into the CNVM and to provide long-term expression of the transferred β-gal cDNA.

**CONCLUSIONS.** The feasibility of gene transduction targeted to the photocoagulation-induced CNVM was demonstrated using retroviral vectors. By transducing functional genes, this model could be useful for investigating the possibility of gene therapy to inhibit formation of the CNVM in age-related macular degeneration. (Invest Ophthalmol Vis Sci. 1998;39:2474-2478)

**MATERIALS AND METHODS**

**Retroviral Vectors**

The PA317 packaging cells were used to generate β-gal expression vectors G1NβSvNa (G1, MoMuLV LTR; βN, nuclear target β-Gal construct, Sv, SV40 early region enhancer/promotor; Na, neo gene) and G1XSvNa (containing only the SV40 promoter-driven neo gene) (Genetic Therapy, Inc., Gaithersburg, MD) at 6 × 10⁶ colony forming unit (cfu)/ml. These vectors were concentrated by ultracentrifugation to a titer of 1 × 10⁸ cfu/ml after collection in UltraDOMA (Biowhittaker, Walkersville, MD) serum-free medium. All producer cell lines tested negative for replication-competent virus.

**Pigmented Rats**

Thirty-five male pigmented rats (Brown Norway) were used in the present study (Jackson Laboratories, Bar Harbor, ME). All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the University of Southern California Institutional Care and Use Committee. The rats were anesthetized with an intraperitoneal injection of a mixture of ketamine hydrochloride (24 mg/kg) and xylazine hydrochloride (2 mg/kg). The pupils were dilated with 1% tropicamide.

**Time Course of β-Galactosidase Expression in Laser-Induced Lesions**

Twenty-three pigmented rats received 10 laser burns (75-μm spot size, 0.1-second duration, 100 mW) in their right eyes. Two days later, 20 μl G1NβSvNa (20 rats) or G1XSvNa (3 rats) was administered into the vitreous cavity of the right eye of each animal, using a 30-gauge needle under a dissecting microscope. One eye each (days 3 and 5) or three eyes each (1 and 2 weeks and 1, 2, 3, and 4 months) were examined for β-gal expression by histochemical staining using the β-gal substrate X-Gal. The retinas were examined using a dissecting microscope, and the number of the CNVMs induced by photocoagulation with or without β-gal expression was counted. Control eyes were examined at 1 and 2 weeks and 1 month for β-gal expression.

**Relation between Fluorescein Leakage and β-gal Expression in Laser-Induced Lesions**

Three burns of a diode laser (75-μm spot size, 0.1-second duration, 100 mW) were delivered to each retina inferior to the optic disc through a slit lamp in the right eyes. Two days later, vector administration was performed. The 14 rats were divided into two groups (7 rats per group) according to the route of vector supernatant administration. In the subretinal injection group, after paracentesis, 20 μl G1NβSvNa (6 rats) or G1XSvNa (1 rat) was injected into the subretinal space from the scleral side using 30-gauge needles. Successful subretinal injection was confirmed by indirect ophthalmoscopy showing clear vitreous and bullous retinal detachment. In the intravitreal injection group, 20 μl G1NβSvNa (6 rats) or G1XSvNa (1 rat) was administered into the vitreous cavity of the right eye of each animal. Fluorescein angiography was performed in all rats at 1 and 2 weeks. All rats were killed, and β-gal expression in the lesions was examined by X-Gal staining.
Histopathologic Analysis
After macroscopic examination, the eyes stained for β-gal were used for histopathologic examination. The sections were examined by light microscopy, and the nuclear β-gal expression was detected. Counter staining was performed with nuclear-fast-red stain.

Transduction Efficiency
In the subretinal injection group, the number of transduced (blue) and nontransduced (red) cells in seven CNVM, which showed β-gal expression, was counted to determine the transduction efficiency (i.e., the ratio of transduced cells to total cells).

RESULTS
Time Course of β-gal Expression in Photocoagulation-Induced Lesions
The number of β-gal-positive photocoagulation-related lesions, induced by intravitreal injection of the vector, was counted and compared with the total number of lesions made. β-gal expression was observed in 0% (days 1 and 3), 16.2% ± 6.8% (day 5), 21.3% ± 9.5% (1 week), 22.9% ± 13.8% (3 weeks), 17.4% ± 8.5% (1 month), 11.8% ± 7.9% (2 months), 5.6% ± 4.8% (2 months), and 3.7% ± 2.4% (4 months). Under the dissecting microscope, the blue reaction product indicating β-gal transduction and expression was seen exclusively in the photocoagulation sites (Fig. 1). In control eyes, no β-gal expression was observed.

Relation between CNV Formation and β-gal Expression
CNV was detected in some rats at 1 week, and the incidence increased by 2 weeks. CNV formation was detected by fluorescein angiography in 5 of the 7 β-gal-positive lesions and 7 of 11 β-gal-negative lesions after subretinal injection, and in 1 of 2 β-gal-positive lesions and 8 of 16 β-gal-negative lesions after intravitreal injection (Fig. 2). Although all β-gal-positive cells were present within lesions, there was no statistical association between β-gal positivity and CNV formation within these lesions (Fisher's exact test, \(P = 1.00\)). Therefore, transduction with β-gal does not induce formation of CNV in photocoagulation lesions, and transduction with β-gal does not occur more frequently in lesions that also have CNV. Subretinal injection of vector supernatant resulted in approximately three times as many transduced lesions as intravitreal injection.

Histopathologic Analysis
One day after subretinal injection of vector supernatant, the deeper layers of the retina showed degenerative changes due to photocoagulation damage. The inner layers of the retina from the outer plexiform layer to the inner limiting membrane were well preserved. No β-gal-positive cells were seen. From day 5 to 2 weeks, scattered melanin-laden macrophages with β-gal expression were observed in all layers of the retina (Fig. 3A). From 1 to 4 months, macrophages gradually decreased in number in the retina. At this stage, the CNVM formation became prominent. Clusters of β-gal-positive spindle- to oval-shaped cells were present in these CNVM (Fig. 3B), presumably macrophages, choroidal fibroblasts, and migrating retinal pigment epithelial (RPE) cells. In some eyes with subretinal injection of the β-gal vector supernatant, choroidal distribution of β-gal-positive macrophages was prominent from 2 weeks to 1 month (Fig. 3C). At 2 months, the β-gal-positive cells in the choroid markedly decreased in number, and some of them adhered to the abluminal side of the vascular walls (Fig. 3D). These findings suggested that β-gal-transduced macrophages are cleared from the lesion through the choroidal vasculature. At the edge of the CNVM, regenerative RPE cells maintained their monolayer configuration, and cells expressing β-gal were also observed (Fig. 3E). In the control eyes with empty vector (G1XSvNa) injection, no β-gal expression was observed in the photocoagulation lesions and the overlying retina (Fig. 3F).

Transduction Efficiency
In the subretinal injection group, the cells in the CNVM with β-gal expression had a transduction efficiency of 1.12% ± 0.58% (at 2 weeks).
FIGURE 3. Histochemical staining for β-galactosidase (β-gal) in β-gal transfected (A, B, C, D, E) and control eye (F). (A) A choroidal neovascular membrane (CNVM) induced by intense photocoagulation (2 weeks). Macrophages expressing β-gal (arrows) are located in CNVM and the retina damaged by photocoagulation. Magnification, ×325. (B) A well-formed CNVM (1 month). Some β-gal-positive spindle- to oval-shaped cells (arrows) are present in the CNVM. Magnification, ×750. (C) The choroid adjacent to CNVM (1 month). Scattered β-gal-positive cells are observed in the eyes with a subretinal injection of β-gal vector supernatant (arrows). Judging from their morphology, these cells are macrophages. M, CNVM. Magnification, ×750. (D) The choroidal vessels adjacent to CNVM (2 months). A β-gal-positive cell, presumably a macrophage, is attached to the abluminal surface (arrow). Magnification, ×750. (E) The edge of CNVM at 2 weeks. There is a regenerative retinal pigment epithelial (RPE) cell expressing β-gal (arrow). These RPE cells at the edge of photocoagulation lesions divide and migrate to cover the denuded area. Magnification, ×750. (F) A CNVM in a control eye with empty vector injection (2 weeks). There is no β-gal expression present. Magnification, ×750.

DISCUSSION

In the present study, we demonstrated the feasibility of exogenous gene transfer to CNVM that was induced by laser photocoagulation. This model has several features that are advantageous for investigating the possibilities of gene therapy for CNV. The time course of CNV formation and the expression of the β-gal gene in the CNVM were appropriate to determine the effects of transferred genes on the development of CNV. CNV was detected in some rats at 1 week, and the incidence increased at 2 weeks; fluorescein leakage was still observed at 4 months in some eyes (data not shown at 4 months). β-gal expression was first detected at 1 week in the laser lesions and was still detected at 4 months. This long-term expression of the transferred gene is probably necessary to produce a therapeutic effect on CNV, which is chronic in nature. This model is
also suitable for gene therapy studies because CNV formation and β-gal gene expression were consistent and reliable. CNV could be identified in approximately 50% of the lesions at 2 weeks by fluorescein angiography. β-gal expression was detected in the photocoagulation-induced lesions from day 7 (26.8% ± 8.1%) to 4 months (3.7% ± 2.4%). Although not remarkably high, these numbers appear to be sufficiently high to allow detection of the effects of transferred genes on CNV formation. Rats are relatively inexpensive and multiple CNV can be induced by laser photocoagulation in one eye. In addition, the procedures required to induce CNV are well standardized. Therefore, it is possible to generate a large number of lesions, allowing statistical analysis of the effect of gene transfer on CNV development.

As demonstrated in the present study, β-gal transduction and expression were restricted to the CNVM. This selectivity may reflect the fact that retroviral vectors transduce the β-gal construct into dividing cells in the CNVM but not in normal retinochoroidal tissue. There is minimal cell division in the normal adult retina. Because the CNVM in AMD is located near the fovea, which is important for central vision, this selectivity provides another advantage for use of retroviral vectors. We could thus reduce the chance of unwanted gene transfer to the retina, especially in the sensitive fovea. This selectivity cannot be obtained by other types of vectors that transfer exogenous genes into quiescent and dividing cells.7

Because the CNVM formation in AMD can be interpreted as an abnormal cell proliferation in the submacular region, one potential strategy to inhibit CNVM development is the selective elimination of proliferating cells from the CNVM. We previously reported an experimental gene therapy to treat preretinal membranes in rabbits.8 Proliferating fibroblasts on the retinas were selectively killed by transducing the herpes simplex virus thymidine kinase (H-tk) gene followed by ganciclovir administration. In this experiment, only 1% of cells transduced with H-tk gene in the preretinal membrane was of value in reducing the severity of proliferative vitreoretinopathy, owing to the bystander effect.8 In the present study, we obtained a transduction efficiency of 1.12% ± 0.58% in the CNVM in the subretinal injection group at 2 weeks. Consequently, a similar H-tk gene strategy could be used to treat CNVM in eyes with AMD.

Another strategy to inhibit CNV is to transduce cDNAs coding diffusible anti-angiogenic factors into CNVM and adjoining regenerative RPE cells. As demonstrated in the present study, long-term expression of the anti-angiogenic factors should be obtained in the lesion. Because a gene defect involving tissue inhibitors of metalloproteinases-3 is known to be present in Sorsby’s fundus dystrophy, which is characterized by marked CNV formation,10 cDNA of TIMP is one potential candidate to be used in this strategy. Antisense against vascular endothelial growth factor or basic fibroblast growth factor is also promising, because expression of these two growth factors has been shown in surgically excised CNVM.3.4 In our previous study, retroviral vectors could transduce the β-gal cDNA into photocoagulation lesions delivered at the parameters comparable to those used clinically to occlude CNV (200 μm, 200 mW, 0.2 second).9

Subretinal injection of vector supernatant containing the β-gal cDNA provided better transduction efficiency into the CNVM than intravitreal injection. However, subretinal injection of vector supernatant after photocoagulation therapy cannot be performed on an outpatient basis. Subretinal injection of the vector supernatant could be used to inhibit the recurrence of the CNVM after their surgical removal. Because cell proliferation should occur in the repair process of the lesion, the H-tk gene could be transduced by injecting retroviral vectors carrying H-tk gene into the subretinal space at the time of surgery. During the postoperative follow-up, if the cell proliferation in the lesion were to become excessive and recurrence of the membrane becomes likely, ganciclovir could be injected into the vitreous cavity to initiate the inhibition of cell proliferation. Alternatively, a retroviral vector coding for an anti-angiogenic protein could be used to prevent regrowth of adjacent endothelial cells.

The photocoagulation-induced CNV model has proven to be a feasible system to investigate the possibility of gene therapy for CNV in clinical practice. Transduction of anti-angiogenic cDNAs in this model could provide important information concerning the possibility of gene therapy for AMD.

References